

large to enable statistical analyses to verify the significance of differences in tumor marker expression for disease progression.

Indices as provided by the invention can also be constructed using any relevant tumor marker associated with disease progression, again provided that there is available a representative cohort of samples of the tumor type having varying degrees of tumor invasiveness and metastatic spread, to enable the production of a weighted scale of expression levels of the three tumor markers. Preferably, the size of the cohort is sufficiently large to enable statistical analyses to verify the significance of differences in tumor marker expression for disease progression. Additional tumor markers can be added to the three tumor markers used in the practice of this invention, or other tumor markers can replace any of the markers described herein, if such markers meet the proviso discussed above.

The methods of the invention are practiced by determining expression levels of the three preferred tumor markers (p53 nuclear accumulation, thrombospondin-1 expression and microvascularization) in a human cancer patient sample. In preferred embodiments, expression levels are determine immunohistochemically. However, expression levels can be determined using any appropriate and convenient method. For example, *in situ* polymerase chain reaction (CITE) and *in situ* nucleic acid hybridization methods for determining expression levels of TSP-1 fall within the methods of the invention. Additionally, site-specific mutation analysis, including sequence analysis or mutant allele-specific amplification of mutant p53, can be used for determining expression levels of mutant p53 in a tumor sample. Similarly, any method for detecting microvascularization, including any method of specific staining, fall within the ambit of the methods of the present invention. Detection methods are chosen appropriate for the labeling or identification of any of the three tumor markers used in the practice of the invention.

In a preferred embodiment, the present invention uses immunohistochemical methods for detecting expression levels of the tumor markers of the invention. In the practice of the invention, antibodies or antisera, preferably polyclonal antisera, and most preferably monoclonal antibodies specific for each marker are used to detect expression levels, using anti-p53, anti-TSP-1 and anti-CD31 antibody immunostaining. Detection of these antibodies can be realized by direct labeling

of the antibodies themselves, with labels including a radioactive label such as ^3H , ^{14}C , ^{35}S , ^{125}I or ^{131}I , a fluorescent label, a hapten label such as biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal antibody specific for the primary antibody. In a preferred embodiment, the primary antibody or antisera is unlabeled, the secondary antisera or antibody is conjugated with biotin and enzyme-linked streptavidin is used to produce visible staining for histochemical analysis.

Detection and quantitation of the tumor markers is provided using methods appropriate for the staining or other detection method used. In preferred embodiments, immunohistochemically stained sections of a tumor sample are analyzed microscopically, most preferably by light microscopy of a sample stained with a stain that is detected in the visible spectrum, using any of a variety of such staining methods and reagents known to those with skill in the art. Most preferably the methods of the invention are practiced by those with skill in the histological arts, but embodiments of the invention provided to permit relatively unskilled technicians to properly interpret tumor marker results are also within the scope of the methods provided.

The following Examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature.

EXAMPLE 1

Tumor Progression/Prognosis Analysis for Breast Cancer

Nuclear localization of p53 protein, thrombospondin 1 expression levels and extent of microvascularization were determined immunohistochemically as follows.

Tumor blocks from breast cancer patients were obtained from Western Medical Center and H. Lee Moffitt Cancer Center and examined independently by two pathologists to confirm the diagnosis for tumor type and stage. Representative section of each tumor sample were chosen on the basis of pathological examination for immunohistochemical staining. Tissue sections 5 microns in thickness were cut and prepared on slides using standard histological preparation techniques. Since paraffin sections were used, slides were first deparaffinized using HistoClear

(Biogenics, California). Antigens were exposed for immunohistochemical staining by pronase digestion (for CD31 detection) and by microwave boiling (for p53 and thrombospondin 1 (TSP-1) detection) using antigen recovery solution (Biogenics). Slides were then incubated in a solution of 3% hydrogen peroxide in distilled water at room temperature for 10 min, then rinsed briefly with water. Slides were then incubated for 10 min at room temperature using 100 μ L goat serum as blocking buffer. Excess blocking buffer was removed from the slides by shaking, and the slides then incubated with primary antibody at room temperature for 30 min. The primary antibodies used in these assays were: antibody DO1 for p53 (obtained from Santa Cruz Biotech, Santa Cruz, CA); antibody clone 12 for TSP-1 (Immunotech, Inc., Westbrook, ME); and an endothelial cell-specific antibody reactive with the cell surface antigen CD31 for microvascularization (Dako, Carpinteria, CA). Slides were rinsed twice with phosphate buffered saline (PBS) for 5 min after primary antibody incubation.

For detection of primary antibody binding, tissue sections were then incubated with biotinylated goat antimouse immunoglobulin for 20 min at room temperature in a humidified chamber (70-100% relative humidity). Slides were rinsed twice with PBS after this incubation, and then treated with a solution of peroxidase-conjugated streptavidin for 20 at room temperature. After being rinsed again with PBS, the slides were incubated in a solution of 3,3'-diaminobenzidine for 3 min at room temperature. Slides were rinsed with PBS for 5 min, exposed to hematoxylin for 1 min, rinsed with water for 10 min, dehydrated in an ascending ethanol series, cleared with xylene, mounted and viewed by light microscopy.

Microscopic analyses were performed at 200X magnification as follows. The malignant cells on the slide were counted, and the number of stained cells and staining intensity determined. Each slide was scored independently by two pathologists. Scoring of staining intensity was relative to the following scale:

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| 0 | = | staining intensity equal to the negative control |
| 1 | = | staining intensity weak but greater than negative control |
| 2 | = | staining intensity moderate (more than negative control, but less than positive control) |